AD-A175 272

DTIC ACCESSION NUMBER

LEVEL

PHOTOGRAPH THIS SHEET

STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXIN ANNUAL SUMMARY REPORT

FEB. 4, 1986

DOCUMENT IDENTIFICATION

1

INVENTORY

DISTRIBUTION STATEMENT A
Approved for public release;
Distribution Unlimited

DISTRIBUTION STATEMENT

ACCESSION FOR		
NTIS	GRA&I	☑
DTIC	TAB	☐
UNANNOUNCED		☐
JUSTIFICATION		
BY		
DISTRIBUTION /		
AVAILABILITY CODES		
DIST	AVAIL AND/OR SPECIAL	
A-1		

DISTRIBUTION STAMP

DTIC
ELECTE
DEC 23 1986
D

DATE ACCESSIONED

DATE RETURNED

DTIC FILE COPY

86 12 ... 041

DATE RECEIVED IN DTIC

REGISTERED OR CERTIFIED NO.

PHOTOGRAPH THIS SHEET AND RETURN TO DTIC-FDAC

AD _____

STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXIN

Annual Summary Report
September 15, 1984-September 14, 1985

B. R. DasGupta

February 4, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-84-C-4245

University of Wisconsin
Madison, Wisconsin 53706

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION / AVAILABILITY OF REPORT Approved for public release; distribution unlimited
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION Food Research Institute 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code) University of Wisconsin-Madison 1925 Willow Drive Madison, Wisconsin 53706	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research & Development Command 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-84-C-4245
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3M1-62770A871; TASK NO. AB; WORK UNIT ACCESSION NO. 362

11. TITLE (Include Security Classification)

(U) STUDY OF TOXIC AND ANTIGENIC STRUCTURES OF BOTULINUM NEUROTOXINS

12. PERSONAL AUTHOR(S)
DasGupta, B.R.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual	FROM 9/15/84 TO 9/14/85	1986, February 4	7

16. SUPPLEMENTARY NOTATION

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Type B strain 657, role of lysine and tyrosine residues, toxoid (second generation), tertiary structure, enzymatic cleavage of heavy chain, amino acid sequence of the two OVER
[illegible]	01		
[illegible]	20		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The neurotoxin (NT) from type B, strain 657, has been partially purified. Lysine and tyrosine residues in types A and E NT were selectively modified to study their roles in toxicity and antigenicity. A large number of lysine residues were not critical for toxicity. Integrity of tyrosine residue(s) were critical for toxigenic structures but not for serological reactivity. Type E NT completely detoxified following modification of tyrosine residues proved to be a good immunogen (second generation toxoid). Antiserum raised in rabbits neutralized the NT. The heavy chain of type A NT was enzymatically fragmented into two halves and partially sequenced. The conformation of the single chain type E NT appeared more unfolded when it was nicked to the dichain form.

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	UNCLASSIFIED
22a. NAME OF RESPONSIBLE INDIVIDUAL Mrs. Judy Pawlus	22b. TELEPHONE (Include Area Code) 301/663-7325
22c. OFFICE SYMBOL SGRD-RMI-S	

DD FORM 1473, 84 MAR — 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE

Block Number 18 - Cont.

halves of heavy chain.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

SUMMARY

A number of sequential steps have been instituted for the purification of the neurotoxin (NT) produced by type B strain 657. It appears that this NT cannot be purified by simply following the various steps required for purifying the NT from type B strain Okra. In the areas of structure and function relationship our studies progressed in four areas: i) The heavy chain of type A NT was enzymatically cleaved into two halves. These fragments were purified and partially sequenced. ii) Trypsin nicks the single chain type E NT to the dichain protein mediating over 100-fold activation. We searched for a difference in the tertiary structure between the single and dichain forms; the dichain (activated form) protein had more unfolded conformation than the single chain protein. iii) Lysine and tyrosine residues in types A and E NT were selectively modified to study their roles in toxicity and antigenicity. A large number of lysine residues were not critical for toxicity. Integrity of tyrosine residue(s) were critical for toxigenic structures but not for serological reactivity. iv) Type E NT completely detoxified following modification of tyrosine residues proved to be a good immunogen. This toxoid (second generation toxoid) given to rabbits elicited antiserum that had high NT neutralizing titer.

BODY OF REPORT (TEXT)

Research activities under the contract "study of toxic and antigenic structures of botulinum neurotoxin" covered areas of all five specific aims of the contract.

1. PURIFICATION OF TOXIN FROM TYPE B STRAIN 657

C. botulinum type B strain 657 stock culture was obtained from Dr. C. Hathaway, CDC. We attempted to produce toxin in large volume of toxin producing media following the well established procedure of production of type B toxin from type B strain Okra. Yield of toxin was very poor, not adequate to proceed through various steps of purification. The stock culture was sent to Dr. Lynn Siegel, Fort Detrick for sub-culturing and isolation of colonies more toxigenic than the parent culture. We have received such isolates. In the meantime we have established few sequential steps e.g. acid precipitation of the toxin from the toxin producing media following 5-7 days of culturing, digestion of the precipitated toxin with RNase, passage of the digest through DEAE-Sephadex A-50 column (pH 5.5, 0.05 M citrate buffer). These steps, that substantially purified the toxin, we believe will be parts of the procedure to be eventually designed to completely purify the toxin. It appears that the toxin from strain B 657 cannot be purified by simply following the various steps required for purifying the toxin from strain Okra.

2. STRUCTURE-FUNCTION RELATIONSHIP

Our objective is to determine the role of different functional groups of the amino acid residues.

Lysine: The amino groups of type A neurotoxin (NT) were modified by means of reductive methylation at two different molar ratios of protein to reagent. The protein modified with the higher reagent concentration lost ~80% toxicity (mouse lethality). Amino acid analysis of the modified proteins are underway. No amino acid residue other than lysine appears to have been modified. The number of lysine residues modified is being determined as mono-, di- and trimethylated lysine, rather than loss of lysine compared to the control (native protein). Separation of the three derivatives using an amino acid analyzer requires special conditions. The number of lysine residues modified will be related to loss in toxicity.

Tyrosine: The first phase of our experimental work on the role of tyrosine residues in the biological activities of types A and E NT is now complete. Tyrosine residues of the two NT types were modified with tetranitromethane at different protein to reagent molar ratios. Reagent dose-dependent modification and detoxification of the proteins were observed with no or very little damage to serological reactivity. Amino acid analysis has confirmed formation of nitrotyrosine without the modification of other residues. We infer that integrity of tyrosine residue(s) is critical for toxigenic structures but not for serological reactivity (against polyclonal antibody).

3. SECOND GENERATION TOXOID

The type E NT completely detoxified by tetranitromethane was used as a toxoid to raise antibody in rabbits. The toxoid was prepared twice separately from two different batches of the NT. Each batch of toxoid was administered to two rabbits. The antiserum neutralized the NT (mouse lethality); lethality neutralizing titer of the serum was at least 9.8×10^5 LD_{50}/mL or 98 International Units/mL.

We consider this toxoid as a second generation toxoid (first generation toxoid being a crude preparation of NT detoxified with formalin) that has the following noteworthy features: i) Prepared with pure NT, its ii) chemical modification is more specific than the products that form over 7-10 days of reaction between NT and formaldehyde, and iii) it is more homogeneous, electrophoretically, than pure NT detoxified with formaldehyde. iv) Also, the covalent modification is irreversible, hence the toxoid cannot undergo spontaneous reversal to the toxic form. This last point is important because diphtheria and tetanus toxins detoxified with formaldehyde (to prepare toxoid for immunization) are known to revert back to the toxic form (Akama, K., Ito, A., Yamamoto, A., and Sadahiro, S. Jap. J. Med. Sci. Biol. 24, 181 [1971]).

4. ALTERATION OF TERTIARY STRUCTURE OF NT DUE TO MODIFICATION

Chemical modifications of amino acid residues of a NT or enzymatic cleavage might alter the tertiary structure of the NT. The associated altered biological activity might not result directly from the modification or fragmentation but might result from change in the conformation. No experimental data on the conformations of types A, B or E NT is available. Antigenically distinct NT types A and E isolated from the bacterial cultures are fully active dichain and mildly active single chain proteins, respectively. Trypsin nicks the single chain to the dichain protein mediating over 100 fold activation. We searched for a difference in the tertiary structure between the single and dichain forms.

We initiated these studies with types A and E NT, using a fluorogenic probe 2,6-toluidinylnaphthalenesulfonic acid (TNS). This dye was better than 8-anilino-1-naphthalene sulfonate for our purposes. The fluorescence of TNS was strongly enhanced in the presence of NT. Emission of TNS bound to NT was maximal at 428 nm, although TNS by itself did not have this property. Titrations with TNS at two widely different pHs show that for a given protein concentration of dichain type A or single chain type E NT the fluorescence was 1.7 to 3.0 more intense at pH 9.0 than at pH 6.0; apparently the two NT types at pH 9.0 have more unfolded structures that allow more binding between TNS and protein. At pH 6.0 TNS produced higher fluorescence in the presence of dichain than with single chain type E NT. The dichain type E NT apparently binds more TNS than the single chain form because of its more unfolded conformation. This is the first experimental demonstration that nicking of type E leads to conformational change at the level of tertiary structure.

5. ENZYMATIC CLEAVAGE OF THE H CHAIN AND PARTIAL SEQUENCE OF THE TWO FRAGMENTS

The type A NT isolated from Clostridium botulinum culture is a dichain protein. The H and L chains (M_r 97K and 53K, respectively) are linked by at least one -S-S- bond. We have cut the H chain near the middle (trypsin:NT, 1:10 (w/w), pH 6.0, 30°, 10C min). The cleavage products are: i) L chain linked to one half of the H chain (M_r ~50K) by a -S-S- bond (hence total M_r ~103K), and ii) the other half (M_r ~47K) of the H chain not linked to the other two polypeptides via a -S-S- bond. The two halves of the H chain were purified by ion exchange chromatography. The first 27 residues of one piece (M_r ~50K) of the H chain were identified. Following sequence data from two independent preparations matched:
A.L.N.D.L.C.I.K.V.N.N.W.D.L.F.F.S.P.S.E.D.N.F.T.N.D.L.-. This sequence is identical to the N-terminal sequence of the intact H chain (BBRC 119, 900). Two points emerge: i) We now have 17 additional residues of the H chain sequenced (compared to the first 10 of the intact H chain that were known), and ii) the -SH of the H chain that forms the -S-S- link with the L chain is situated on the N-terminal half of the H chain. We have also partially sequenced the other half (M_r ~47K) of the H chain (result of one run):
Y.I.I.N.L.X.I.L.N.L.R.Y.-.

DISTRIBUTION LIST

1 Copy: Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMS
Fort Detrick, Frederick, Maryland 21701-5012

5 Copies: Commander
US Army Medical Research Institute of Infectious Diseases
ATTN: SGRD-UIZ-M
Fort Detrick, Frederick, MD 21701-5011